*Br. J. Pharmacol.* (1992), **107**, 544–552 

# Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin

[1]S. Bevan, S. Hothi, G. Hughes, I.F. James, H.P. Rang, K. Shah, C.S.J. Walpole & J.C. Yeats

Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN

**1** Capsazepine is a synthetic analogue of the sensory neurone excitotoxin, capsaicin. The present study shows the capsazepine acts as a competitive antagonist of capsaicin.

**2** Capsazepine (10 μM) reversibly reduced or abolished the current response to capsaicin (500 nM) of voltage-clamped dorsal root ganglion (DRG) neurones from rats. In contrast, the responses to 50 μM γ-aminobutyric acid (GABA) and 5 μM adenosine 5'-triphosphate (ATP) were unaffected.

**3** The effects of capsazepine were examined quantitatively with radioactive ion flux experiments. Capsazepine inhibited the capsaicin (500 nM)-induced $^{45}Ca^{2+}$ uptake in cultures of rat DRG neurones with an $IC_{50}$ of 420 ± 46 nM (mean ± s.e.mean, $n = 6$). The $^{45}Ca^{2+}$ uptake evoked by resiniferatoxin (RTX), a potent capsaicin-like agonist was also inhibited. (Log concentration)-effect curves for RTX (0.3 nM–1 μM) were shifted in a competitive manner by capsazepine. The Schild plot of the data had a slope of 1.08 ± 0.15 (s.e.) and gave an apparent $K_d$ estimate for capsazepine of 220 nM (95% confidence limits, 57–400 nM).

**4** Capsazepine also inhibited the capsaicin- and RTX-evoked efflux of $^{86}Rb^+$ from cultured DRG neurones. The inhibition appeared to be competitive and Schild plots yielded apparent $K_d$ estimates of 148 nM (95% confidence limits, 30–332 nM) with capsaicin as the agonist and 107 nM (95% confidence limits, 49–162 nM) with RTX as agonist.

**5** A similar competitive inhibition by capsazepine was seen for capsaicin-induced [$^{14}$C]-guanidinium efflux from segments of adult rat vagus nerves (apparent $K_d = 690$ nM; 95% confidence limits, 63 nM–1.45 μM). No significant difference was noted in the apparent $K_d$ estimates for capsazepine in assays on cultured DRG neurones and vagus nerve as shown by the overlap in the 95% confidence limits.

**6** Capsazepine, at concentrations up to 10 μM, had no significant effects on the efflux of $^{86}Rb^+$ from cultured DRG neurones evoked either by depolarization with high (50 mM) $K^+$ solutions or by acidification of the external medium to pH 5.0–5.6. Similarly capsazepine had no significant effect on the depolarization (50 mM KCl)-induced efflux of [$^{14}$C]-guanidinium from vagus nerve preparations.

**7** Ruthenium Red was also tested for antagonism against capsaicin evoked [$^{14}$C]-guanidinium release from vague nerves and capsaicin induced $^{45}Ca^{2+}$ uptake in cultures of DRG neurones. In contrast to capsazepine the inhibition by Ruthenium Red (10–500 nM in DRG and 0.5–10 μM in vagus nerve experiments) was not consistent with a competitive antagonism, but rather suggested a more complex, non-competitive inhibition.

**Keywords:** Capsaicin; resiniferatoxin; Ruthenium Red; capsazepine; sensory neurones

## Introduction

Capsaicin, the pungent ingredient in hot chilli peppers, has a unique excitatory action on a sub-population of afferent sensory neurones. When capsaicin interacts with these neurones a cation selective ion channel is opened (Wood *et al.*, 1988; see review by Bevan & Szolcsanyi, 1990). This allows sodium and calcium ions to enter and potassium ions to leave the cell. The net effect is an inward current that depolarizes and thus excites the neurones. Not all sensory neurones are depolarized by capsaicin; the chemosensitivity is restricted to some somatic and visceral afferents with conduction velocities in the C- and Aδ-range. The capsaicin-sensitive somatic neurones include the polymodal nociceptors, which are sensitive to a variety of noxious chemical, thermal and mechanical stimuli, while the visceral neurones mediate important autonomic reflexes (see Szolcsanyi, 1990; Holzer, 1991). There is also increasing evidence that, in addition to their afferent functions, these neurones have efferent functions in the target tissues possibly as a result of neuropeptides released from the peripheral terminals (see Szolcsanyi, 1984; Holzer, 1988; Maggi & Meli, 1988 for reviews).

In addition to its specific agonist action on sensory neurones, capsaicin has also been reported to inhibit or to modify voltage-gated sodium, potassium and calcium currents in a variety of neurones (Dubois, 1982; Erdelyi & Such, 1984; Yamanaka *et al.*, 1984; Petersen *et al.*, 1987; 1989; Docherty *et al.*, 1991) and also to inhibit contraction of cardiac, visceral and vascular smooth muscle (Szolcsanyi & Bartho, 1978; Donnerer & Lembeck, 1982; Zernig *et al.*, 1984). Other effects on cellular functions such as prostanoid formation (Juan *et al.*, 1980; Flynn *et al.*, 1986) and platelet aggregation (Wang *et al.*, 1984; 1985) have also been reported. The concentration of capsaicin necessary to show these effects is usually higher than that required to show the sensory neurone-specific membrane action, which is postulated to involve specific membrane receptors. Nevertheless such findings raise the possibility that some of the actions of capsaicin could be unrelated to its action on the sensory neurone membrane. The availability of a specific capsaicin antagonist would greatly assist studies on the physiological functions of capsaicin sensitive nerves as well as the mechanisms of action of capsaicin and a related compound, resiniferatoxin (RTX).

Recent studies have demonstrated that RTX, which is a compound isolated from some plants of the genus *Euphorbia*,

[1] Author for correspondence.

has similar effects to capsaicin (de Vries & Blumberg, 1989; Szallasi & Blumberg, 1989; Dray *et al.*, 1990a; Szallasi & Blumberg, 1990a). RTX shows some structural similarities to capsaicin (see Figure 1). Both compounds appear to act on the same neurones, to have a similar, if not identical, mechanism of action (Szallasi & Blumberg, 1989; Winter *et al.*, 1990) and to share a common, membrane associated binding site (Szallasi & Blumberg, 1990b). The difference is that RTX is usually active at 100 to 1000 times lower concentrations than capsaicin (see. e.g. Maggi *et al.*, 1989). It is unclear, however, if all the effects of RTX are due to an action at capsaicin binding sites rather than to activation of protein kinases (cf. Ryves *et al.*, 1989).

The inorganic dye, Ruthenium Red, inhibits the effects of capsaicin on sensory neurones and has been used as a capsaicin antagonist (Maggi *et al.*, 1988a,b; 1989; Amann & Lembeck, 1989; Chahl, 1989; Franco-Cereceda *et al.*, 1989; Dray *et al.*, 1990b). Unfortunately, Ruthenium Red can inhibit the responses to other agents (see Maggi, 1991) and this non-specificity limits its usefulness as a capsaicin antagonist. In this paper we describe the discovery and characterization of a competitive antagonist of both capsaicin and RTX. This antagonist, capsazepine (see Figure 1) has already been shown to antagonize the actions of capsaicin in the spinal cord (Dickenson & Dray, 1991) and will be a valuable tool in future studies on the cellular actions of these agents and the physiological functions of capsaicin-sensitive nerves.

Preliminary reports on the pharmacology of capsazepine have been published (Bevan *et al.*, 1991; Dray *et al.*, 1991).

## Methods

### *Dorsal root ganglion neurone experiments*

*Cell dissociation* Dissociated DRG neurones were prepared from neonatal rats by enzymatic and mechanical dissociation as described in detail elsewhere (Wood *et al.*, 1988). In brief, neonatal animals were killed by decapitation after cervical dislocation. Spinal ganglia were removed aseptically from all levels of the spinal cord. Ganglia were incubated for 30 min at 37°C with F-14 medium (Gibco) containing 0.125% collagenase (Boehringer Mannheim) and 10% horse serum (Gibco) and then for 15–30 min with 0.25% trypsin (Worthington) in F-14 medium. Cells were dissociated by trituration through a flame polished Pasteur pipette, centrifuged and the cell pellet resuspended in F-14 medium containing 10% horse serum and 200 ng $ml^{-1}$ nerve growth factor. Cells were either used immediately for electrophysiology or placed in culture.

For experiments on cultured neurones, dissociated DRG cells were plated either on sterile 13 mm glass coverslips or Terasaki plates. Both substrates were previously coated with 10 $\mu g\ ml^{-1}$ poly-D-ornithine (Sigma) and 5 $\mu g\ ml^{-1}$ laminin (Gibco). Cells were maintained at 37°C in an humidified incubator gassed with 3% $CO_2$ in air.

*Electrophysiology* Experiments were done on dissociated cells shortly (4–12 h) after plating on laminin-coated glass coverslips. DRG neurones were voltage-clamped by the whole cell, tight seal method with an Axopatch 1B amplifier. Wide bore 'patch' pipettes were pulled from 1.5 mm external diameter glass fibre-filled capillaries (Clark Electromedical GC150F) in a 3 stage pull on a P-80 Brown-Flaming micropipette puller (Sutter Instrument Co.). These were fire polished and had a final resistance of 2–5 MΩ when filled. Intracellular, pipette-filling solutions were composed of (mM) KCl 130, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 11, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) 10, pH 7.4. For most experiments pipettes were coated with Repelcote (Sigma) to reduce electrode capacitance.

Recordings were made on the stage of an inverted phase contrast microscope (Nikon Diaphot) in a flow chamber of a design similar to that described by McBurney & Neering (1985). The basic external solution of (mM) NaCl 130, KCl 3.5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5 and sucrose 40, was buffered to pH 7.4 with 10 mM HEPES. Drugs were applied to the cells from a U-tube, which allowed rapid application (< 300 ms) of solutions (Fenwick *et al.*, 1982). All experiments were done at room temperature 20–23°C.

### *Radioactive ion flux experiments*

Efflux experiments were performed on cultures of neonatal rat DRG neurones plated at high density (5000–10 000 per 13 mm diameter coverslip). Either $Rb^+$ or guanidinium ions were studied as both ions are readily permeant through capsaicin activated (Wood *et al.*, 1988), proton activated and depolarization activated (Bevan & Yeats, 1991) channels. The choice of cation was largely a matter of availability. Three to six day old cultures were loaded to equilibrium with either radioactive $Rb^+$ or guanidinium ions by incubation for 2 h at 37°C in medium containing either 2 $\mu Ci\ ml^{-1}$ $^{86}RbCl$ (300 Ci $mol^{-1}$; Amersham) or 10 $\mu Ci\ ml^{-1}$ [$^{14}C$]-guanidine HCl (54 Ci $mol^{-1}$; Amersham). Coverslips were then dipped in non-radioactive medium and mounted in an enclosed chamber (0.5 ml volume), similar in design to that used for electrophysiology. The chamber was perfused at a rate of 1 ml $min^{-1}$ with non-radioactive medium. Cells were perfused for 10 min to remove extracellular radioactivity. The efflux of radioactive ions was then followed by subsequent collection of solution at 1 min intervals. The standard solutions for efflux studies was composed of (mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1 and HEPES 5, titrated to pH 7.4 with NaOH. In experiments designed to investigate the effects of capsazepine on proton-induced permeability changes, the external medium was acidified by replacement of the HEPES buffered solution with a 10 mM MES (2-[N-morphilino]-ethanesulphonic acid) buffered solution (pH 5.0–5.6). Experiments were performed at room temperature (20–23°C). At the end of the experiment, the amount of radioactivity

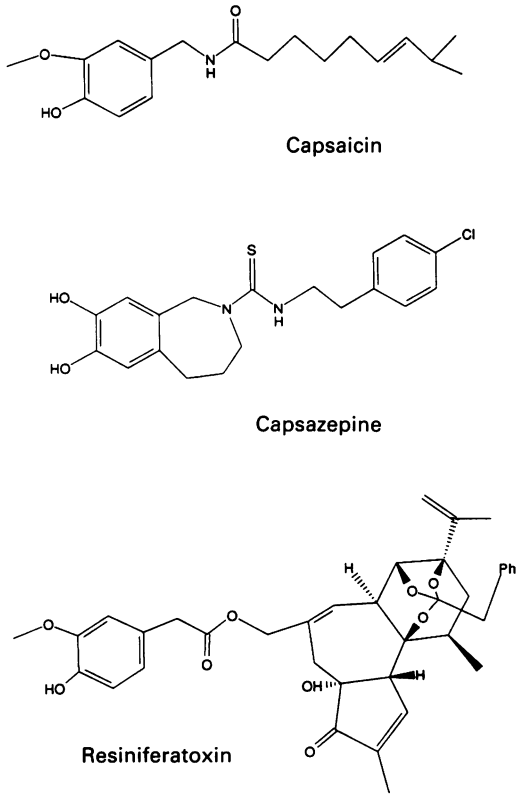


**Figure 1** Structures of capsaicin, resiniferatoxin and capsazepine.

remaining in the cells was determined after solubilization of the cells in 0.2% sodium dodecylsulphate (SDS). The rate of efflux was expressed as a rate constant by calculating the amount of radioactivity released in each 1 min period as a fraction of the amount present at the beginning of the period. The mean efflux for each experimental condition was estimated from at least 4 replicate cultures (usually 5–9). Agonists were applied for 2–3 min and the agonist-induced efflux calculated as the peak increase in efflux rate coefficient.

$^{45}Ca^{2+}$ uptake experiments were carried out on 3–5 day old cultures of DRG neurones plated on 60 well Terasaki plates. The method has been described in detail by Wood *et al.* (1988). In brief, calcium uptake was measured at room temperature over a 10 min incubation period in a nominally calcium-free medium with 10 $\mu$Ci ml$^{-1}$ $^{45}Ca^{2+}$ added. The incubation medium contained varying concentrations of either capsaicin or RTX. The background uptake in the absence of agonist was also determined for each plate. Six replicate wells were used to assess the uptake for each condition tested. At the end of the incubation period the plates were washed with at least 6 changes (10 ml each) of calcium-free medium, the cells were solubilised in 1% SDS and the amount of radioactivity in each sample measured by liquid scintillation counting.

### *Vagus nerve experiments*

Rats (Sprague-Dawley, about 220 g) were killed with chloroform and both cervical vagus nerves removed. The nerves were desheathed and placed overnight at room temperature in 1–2 ml of Locke solution (mM: NaCl 154, KCl 5.6, $CaCl_2$ 2, $NaHCO_3$ 1.8, D-glucose 1.8, Tris-HCl buffer 10, pH 7.4) containing [$^{14}$C]-guanidine HCl (approximately 10 $\mu$Ci ml$^{-1}$). The nerves were then mounted in stainless steel tubes (1 mm internal diameter) through which Locke solution was perfused at a constant rate (1 ml min$^{-1}$) by a peristaltic pump. The emerging solution was collected at 1 min intervals, and the radioactivity was determined by liquid scintillation counting. At the end of the experiments, the nerve was removed, dissolved in 2 N NaOH, and the residual radioactivity was counted. The rate of efflux was expressed as a rate constant. After mounting the nerve and perfusing with Locke solution for 20–30 min, the solution was changed for 5 min to one containing capsaicin or, in some control experiments, 50 mM KCl. In some experiments the preparation was perfused for 5 min with antagonist (capsazepine or Ruthenium Red) alone before changing the solution to one containing capsaicin (or 50 mM KCl) and antagonist. The effect of capsaicin was expressed as the increase in efflux rate constant during the 2 min test perfusion period. Each nerve was tested only once with capsaicin, to avoid problems of desensitization. Each drug combination was tested on 2–4 preparations.

### *Drugs*

Capsazepine (2-[2-(4-chlorphenyl)ethylamino-thiocarbonyl]-7, 8-dihydroxy-2,3,4,5 tetrahydro-1H-2-benzazepine) was synthesized at the Sandoz Institute for Medical Research. Capsaicin, GABA, ATP and Ruthenium Red were obtained from Sigma Chemical Co. Resiniferatoxin was purchased from Fluka.

### *Statistics*

Except where noted, Student's *t* test, modified where appropriate for small sample sizes, was used for statistical analysis of drug effects. Schild plots were constructed from the (log concentration)-response curves by estimating the concentration of agonist to produce a 50% maximum effect. A line was fitted to the Schild plot data by a least squares algorithm (BBN Software Products Corporation, Cambridge, Massachusetts, USA). The asymmetrical confidence limits for the apparent $K_d$ estimates were calculated with Fieller's theorem (see Colquhoun, 1971). Results are given as mean ± s.e.mean.

## Results

### *Electrophysiological studies*

The effects of capsazepine on the chemosensitivity of isolated dorsal root ganglion (DRG) neurones was examined electrophysiologically in voltage-clamped cells. Figure 2a shows the response of an adult rat DRG neurone at a holding potential of −80 mV. Capsaicin (500 nM) evoked an inward current that was reversibly abolished by exposure to a high concentration (10 $\mu$M) of capsazepine. Similar results were observed in 10 other cells examined in this way. The mean amplitude of the current in the presence of capsazepine (0.06 ± 0.03 nA, s.e.mean) was only 4% of that before capsazepine (1.38 ± 0.28 nA). After washing out the capsazepine, the response to capsaicin always increased in size although it usually did not return to the initial level (mean amplitude = 0.71 ± 0.15 nA). This reduction in amplitude after washout of capsazepine probably reflects some degree of desensitization induced by the first application of capsaicin (Yeats *et al.*, 1991). Desensitization made it difficult to make a detailed quantitative study of the effects of capsazepine with electrophysiological methods.

In contrast to the effect on capsaicin-induced currents, 10 $\mu$M capsazepine had no obvious effect on the response to either GABA (Figure 2b) or ATP (Figure 2c). These two agonists were chosen as they evoke reliable and easily studied electrophysiological responses in DRG neurones. The responses to GABA were studied relatively easily as there was little or no desensitization when the drug was applied at 2 min intervals. The mean amplitude of the response to 50 $\mu$M GABA in the presence of 10 $\mu$M capsazepine was 2.12 ± 0.34 nA; this was 1.03 ± 0.08 ($n = 7$) times that of the response to GABA alone.

The effects of capsazepine on ATP responses were less easily studied as ATP responses normally showed a significant degree of desensitization. The responses shown in Figure 2 were atypical as little desensitization was noted in this cell. Nevertheless, they clearly show that 10 $\mu$M capsazepine had no effect on the ATP response. In other experiments different protocols were required. In one set of experiments the ATP sensitivity of the population was sampled either in normal external solution ($n = 14$) or in the presence of 10 $\mu$M capsazepine ($n = 13$). The amplitude of the response in the presence of capsazepine (0.43 ± 0.11 nA) was not significantly different ($P > 0.05$) from that in control solutions (0.37 ± 0.09 nA). The possible effect of capsazepine on the responses to ATP was also studied in single cells by alternating between normal and capsazepine-containing solutions.

In 9 experiments ATP was first applied in normal medium and then in the presence of capsazepine, while in a further 5 experiments ATP was initially applied in capsazepine containing solution and then again 3–4 min after washing out the casazepine. The mean ratio (response amplitude with capsazepine/'control' amplitude) calculated from these experiments was 1.34 ± 0.34, which indicates that capsazepine had no significant effect on the paired responses of individual cells ($P > 0.1$). In view of the large variance of the ratio, these data were also analyzed with a non-parametric test (Wilcoxon sign rank test). This analysis also indicated that capsazepine had no significant ($P > 0.3$) effect on the response of DRG neurones to ATP.

### *Ion flux studies on cultured DRG neurones*

The agonist actions of capsaicin and RTX can be studied quantitatively by following the transmembrane flux of radioactive ions (Wood *et al.*, 1988; Winter *et al.*, 1990). Figure 3

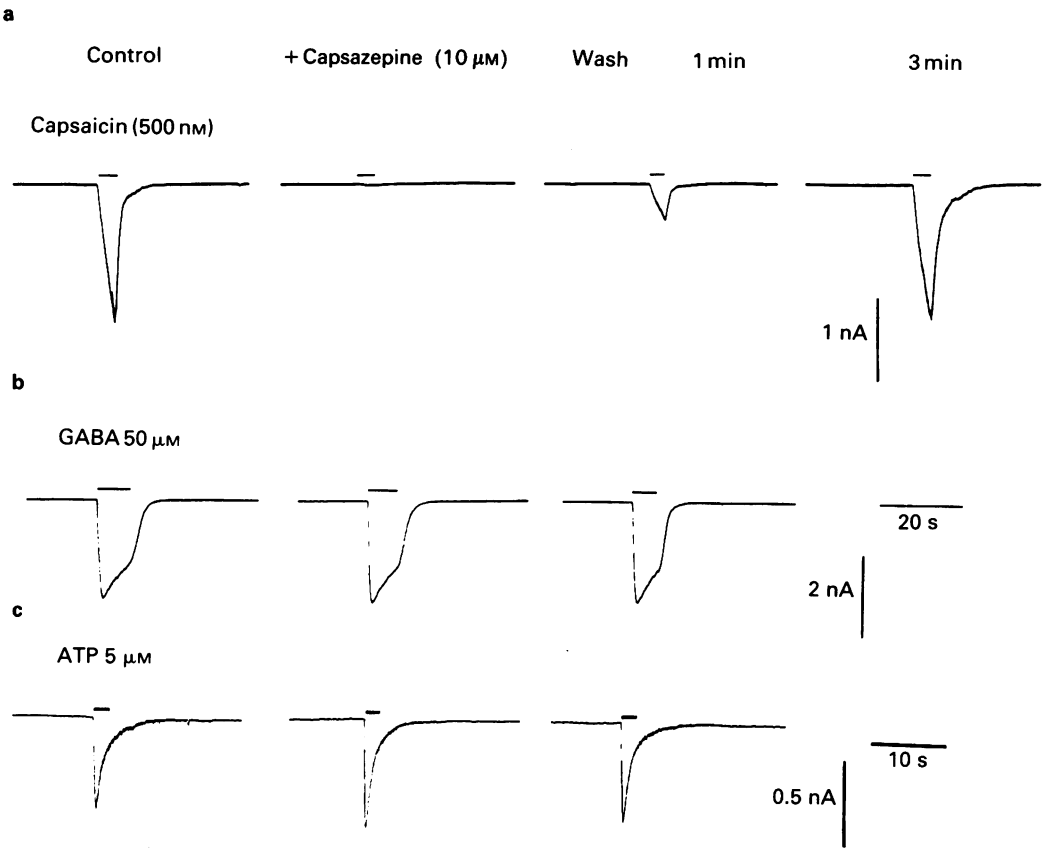


**Figure 2** Effect of 10 μM capsazepine on current responses of neonatal rat DRG neurones evoked by 500 nM capsaicin (a), 50 μM γ-aminobutyric acid (GABA, b) and 5 μM adenosine 5'-triphosphate (ATP, c). Responses measured before, during and after exposure to capsazepine. Each agonist tested on a different cell. Cells were pre-incubated with capsazepine for 3 min before application of each agonist together with capsazepine. For the recovery from capsazepine treatment, responses after two wash times (1 and 3 min) are shown for capsaicin, whereas the responses after 3 min wash periods in capsazepine-free medium are shown for the GABA and ATP experiments. Current calibrations shown for each cell. Time calibration is 20 s for capsaicin and GABA experiments, 10 s for ATP experiment.

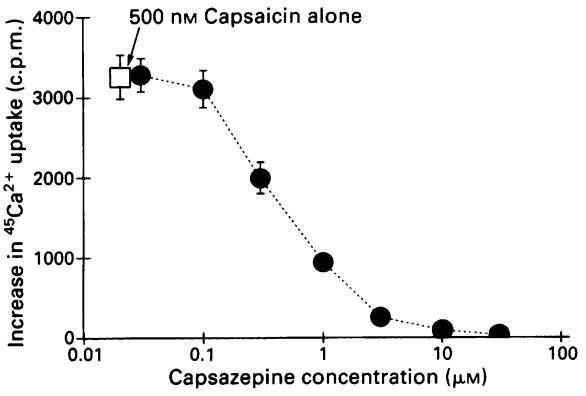


**Figure 3** Inhibition by capsazepine of the uptake of $^{45}Ca^{2+}$ evoked by administration of 500 nM capsaicin to neonatal rat cultured DRG neurones. Data shown as mean with s.e.mean indicated by vertical bars, $n = 6$.

shows the results of a study of the uptake of $^{45}Ca^{2+}$ into cultures of neonatal rat DRG neurones. When tested against 500 nM capsaicin, capsazepine inhibited the uptake with an $IC_{50}$ of 420 ± 46 nM ($n = 6$). Attempts to study this inhibition with capsaicin as the agonist were hampered by the finding that high (⩾ 10 μM) concentrations of capsaicin alone inhibited the $^{45}Ca^{2+}$ accumulation to give a 'bell-shaped' (log-concentration)-effect curve (data not shown). For this reason it was not possible to overcome the capsazepine inhibition by raising the capsaicin concentration. Such experiments were, however, possible with RTX as the agonist because no depression of the maximum $^{45}Ca^{2+}$ uptake was noted even with the highest RTX concentrations used (Figure 4a). RTX induced a concentration-dependent uptake of $^{45}Ca^{2+}$ with an $EC_{50}$ of 3 nM, which is similar to previously published values (Winter *et al.*, 1990). Capsazepine shifted the (log-concentration)-effect curves to the right, but the inhibition was surmountable when the RTX concentration was raised (Figure 4a). No inhibition of the maximum RTX induced $^{45}Ca^{2+}$ was noted in the presence of either 5 μM (3 experiments) or 10 μM (2 experiments) capsazepine ($P > 0.1$ in each experiment). Figure 4e shows the Schild plot constructed from the data in Figure 4a; this had a slope of 1.08 ± 0.15 and yielded a $K_d$ estimate of 220 nM (95% confidence limits, 57–400 nM).

The accumulation of $^{45}Ca^{2+}$ requires several steps including sequestration by intracellular organelles (Wood *et al.*, 1988). Experiments were therefore done to examine the effects of capsazepine on capsaicin- and RTX-induced efflux of $^{86}Rb^{+}$ from cultured DRG neurones, which is a more direct measure of the plasma membrane permeability changes. Capsaicin alone evoked a concentration-dependent increase in $^{86}Rb^{+}$ efflux rate with an $EC_{50}$ of 60 nM (Figure 4c). Increasing concentrations of capsazepine shifted the (log-concentration)-response curves to the right. The Schild plot for these data was linear with a slope of 1.21 ± 0.07 and gave a $K_d$ for capsazepine of 148 nM (95% confidence limits, 30–332 nM, see Figure 4f). RTX also evoked a $^{86}Rb^{+}$ efflux ($EC_{50} = 0.9$ nM) and this was similarly inhibited by capsa-

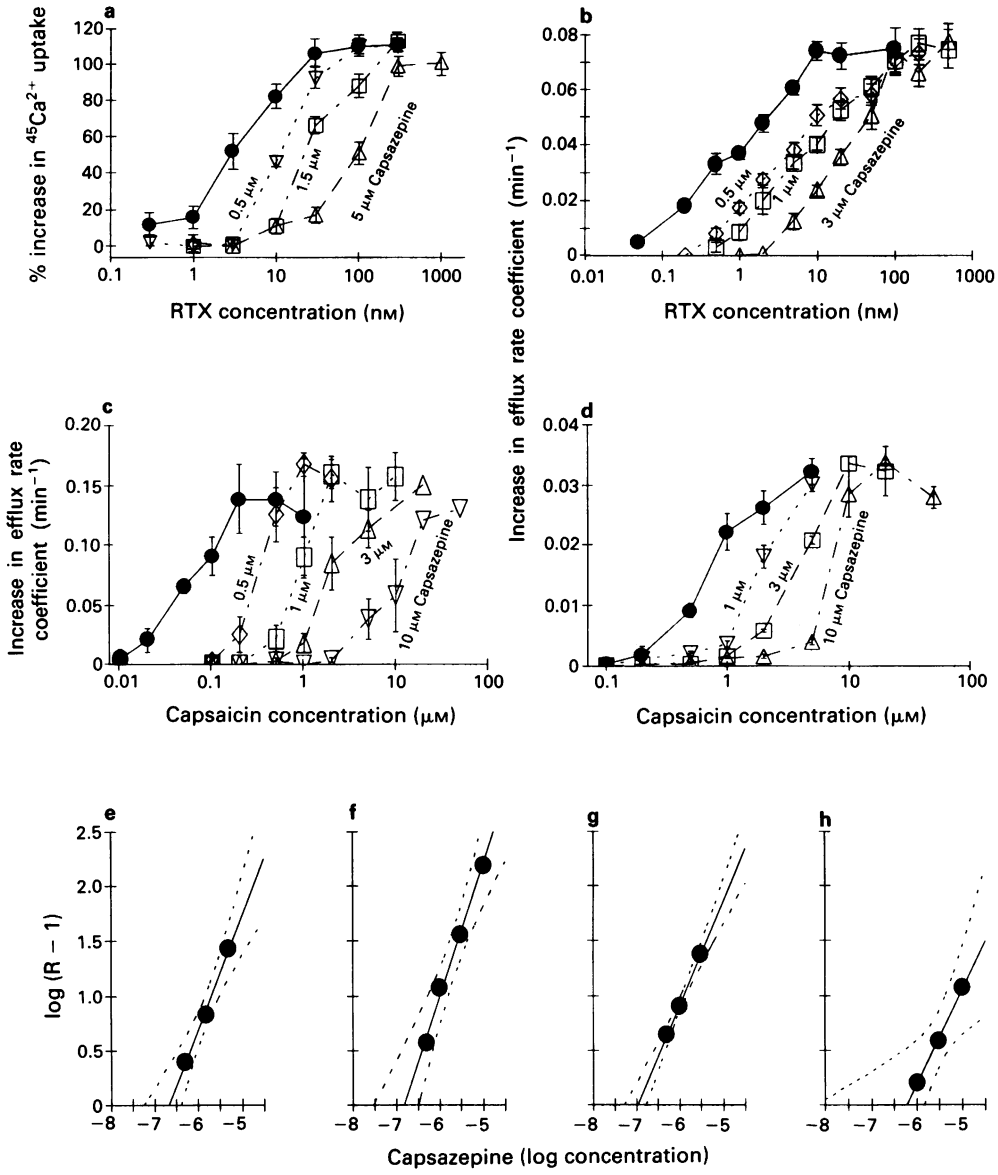


**Figure 4** (a–d) (Log-concentration)-effects curves for capsazepine on (a) resiniferatoxin (RTX)-induced $^{45}Ca^{2+}$ uptake by neonatal rat DRG cultures: (b) RTX-induced $^{86}Rb^{+}$ efflux, cultured DRG neurones: (c) capsaicin-evoked efflux of $^{86}Rb^{+}$, cultured DRG neurones: (d) capsaicin-evoked efflux of [$^{14}$C]-guanidinium from pre-loaded rat vagus nerves. (Log-concentration) – effect curves are shown for agonists alone and in the presence of the indicated concentrations of capsazepine: data shown as mean with s.e.mean shown by vertical bars. (e–h). Schild plots for the data shown in (a–d) respectively. (e) slope = 1.08 ± 0.15, $K_d$ = 220 nM (95% confidence limits, 57–400 nM): (f) slope = 1.21 ± 0.07, $K_d$ = 148 nM (95% confidence limits, 49–162): (g) slope = 0.95 ± 0.03, $K_d$ = 107 nM (95% confidence limits, 30–332 nM): (h) slope = 0.87 ± 0.03, $K_d$ = 690 nM (95% confidence limits, 63 nM–1.45 μM).

zepine with a $K_d$ of 107 nM (95% confidence limits, 49–162 nM) as shown in Figure 4b and g.

An increase in the efflux rate of $^{86}Rb^{+}$ was evoked when cultured DRG neurones were depolarized by a challenge with 150 mM KCl (replacement for NaCl). The potassium evoked $^{86}Rb^{+}$ efflux was not inhibited by 10 μM capsazepine, which was included in the pre-challenge solutions as well as in the high $K^{+}$ solution. The increase in efflux rate coefficient in the presence of capsazepine (0.0418 ± 0.0066 $min^{-1}$, $n = 5$) was not significantly different ($P > 0.1$) from the increase evoked by KCl alone (0.0508 ± 0.0074 $min^{-1}$, $n = 5$).

Acidification of the external medium to pH < 6.4 evokes a sustained inward, depolarizing current in a subset of DRG neurones. The channels responsible for this current are permeable to $Rb^{+}$ and guanidinium ions and so the efflux of either $^{86}Rb^{+}$ or [$^{14}$C]-guanidinium ions from pre-loaded DRG cultures can be used as a measure of the proton evoked response (Bevan & Yeats, 1991). Figure 5 shows the results of such an experiment with $^{86}Rb^{+}$. Acidification of the external medium elicited an increase in the efflux rate, which was not significantly different when 10 μM capsazepine was included in the perfusion medium. Experiments were done with solutions of various pH (5.0–5.6). The ratio (increase in efflux rate in the presence of capsazepine)/(increase in rate for control) was calculated for pH 5.0, 5.2, 5.4 and 5.6 solutions with 4–6 estimates of rate coefficient for each condition. The mean ratio over this pH range was 1.06 ± 0.24 for [$^{14}$C]-guanidinium ($P > 0.1$: total number of preparations: control, $n = 21$; capsazepine, $n = 22$) and 0.98 ± 0.33 for $^{86}Rb^{+}$ ($P > 0.1$, control, $n = 17$; capsazepine, $n = 18$).

### *Ion flux studies on rat vagus nerves*

Radioactive efflux studies were also carried out on vagus nerves from adult rats to examine whether the action of the antagonist was restricted to cells maintained in culture. In

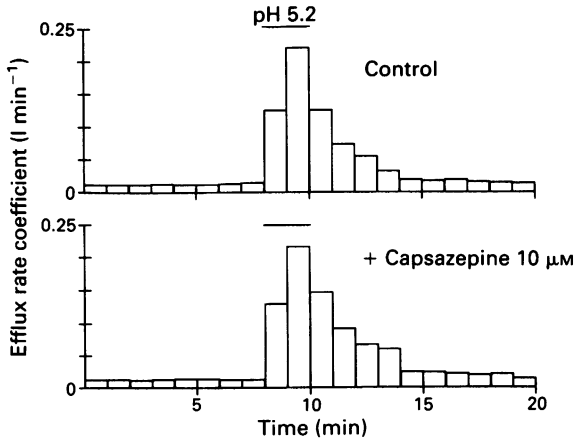


**Figure 5** Lack of effect of 10 μM capsazepine on proton-evoked efflux of $^{86}Rb^+$ from neonatal rat DRG cultures. Each column represents the amount of radioactivity released during a 1 min collection period. Time of application of the acidic solutions shown by bars.

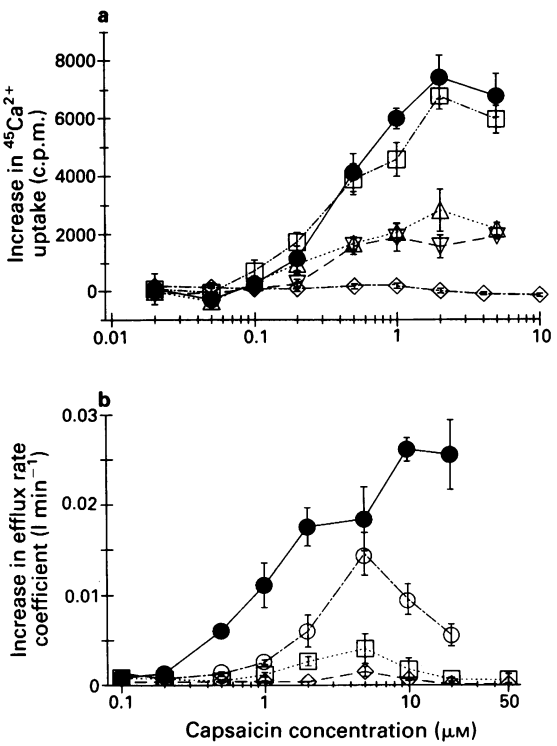


**Figure 6** Effect of Ruthenium Red on capsaicin induced ion fluxes. (Log-concentration)-effect curves. (a) $^{45}Ca^{2+}$ uptake in neonatal rat DRG cultures; Ruthenium Red tested at 5 (□), 25 (△), 50 (▽) and 250 (◇) nM. (b) [$^{14}$C]-guanidinium efflux from adult rat vagus nerves in the absence and presence of 0.1 (○), 0.2 (□) and 5 μM (◇) capsazepine. Preparations pre-incubated with Ruthenium Red for at least 5 min before co-application of agonist. Data shown as mean with s.e.mean indicated by vertical bars.

these experiments [$^{14}$C]-guanidinium ion was used as a convenient permeant cation to study as this, like $Rb^+$, flows through the capsaicin-operated channel (Wood *et al.*, 1988; C.A. Forbes & S. Bevan, unpublished observations). The results were qualitatively similar to those obtained with $^{86}Rb^+$ on cultured DRG neurones. Capsazepine alone, in concentrations up to 10 μM, had no significant effect ($P>0.05$) on the resting efflux rate of guanidinium (control $0.00411 \pm 0.00007$ min$^{-1}$, $n = 31$, 10 μM capsazepine $0.00435 \pm 0.00010$, $n = 19$). However, capsazepine produced a dose-dependent inhibition of the capsaicin-evoked guanidinium efflux that could be surmounted by an increase in the capsaicin concentration (Figure 4d). The Schild plot constructed from the (log-concentration)-effect curves showed a slope of $0.87 \pm 0.03$ (s.e.) and an apparent $K_d$ of 690 nM (Figure 4h; 95% confidence limits, 63 nM–1.45 μM).

Capsazepine was tested for any possible effects on guanidinium efflux elicited by depolarization of the nerve with 50 mM $K^+$. Little or no inhibition was noted over the concentration range 1–10 μM. A challenge with 50 mM KCl alone raised the efflux rate by $0.0087 \pm 0.00052$ min$^{-1}$ ($n = 6$), while similar increases of $0.0082 \pm 0.0054$ min$^{-1}$ ($n = 6$) and $0.0070 \pm 0.0031$ min$^{-1}$ ($n = 10$) were noted in the presence of 1 and 10 μM capsazepine ($P>0.1$).

### *Inhibition of response to capsaicin by Ruthenium Red*

Two sets of experiments were done to investigate the ways in which Ruthenium Red, a reported antagonist, inhibited the actions of capsaicin. The effects of various concentrations of Ruthenium Red on the $^{45}Ca^{2+}$ accumulation by cultured DRG neurones and the capsaicin-induced [$^{14}$C]-guanidinium efflux from vagus nerve are shown in Figure 6a and b. Ruthenium Red inhibited the response to capsaicin in both preparations. However, in contrast to capsazepine, Ruthenium Red flattened the (log dose)-response curves and reduced the maximal responses. No obvious rightward shift in the curves was noted; for example, in the $^{45}Ca^{2+}$ uptake experiments (Figure 6a) the estimated $EC_{50}$ value in control conditions ($0.43 \pm 0.03$ μM) was not significantly different ($P>0.1$) from the estimates in the presence of either 50 nM ($0.29 \pm 0.08$ μM) or 100 nM ($0.32 \pm 0.07$ μM) Ruthenium Red. These data suggest that the inhibition is non-competitive.

## Discussion

The quantitative results of ion flux experiments have shown that capsazepine acts as a competitive antagonist not only for capsaicin but also for the more potent agonist RTX. The slopes of the Schild plots for the experiments on cultured DRG neurones were not significantly different from 1 ($P>0.05$). A similar affinity for capsazepine was estimated from $^{45}Ca^{2+}$ accumulation ($K_d = 220$ nM) and $^{86}Rb^+$ efflux ($K_d = 107$ nM) experiments on cultured DRG neurones when RTX was the agonist. The $^{45}Ca^{2+}$ accumulation assay is a measure of both the influx and the sequestration of $^{45}Ca^{2+}$ and so inhibition could occur at an intracellular site and not at the plasma membrane. In contrast, the efflux experiments are a direct measure of plasma membrane effects. The similarity in the results of both influx and efflux experiments therefore suggests that the inhibition observed in the $^{45}Ca^{2+}$ experiments represents an inhibition at the plasma membrane.

Capsazepine also showed competitive antagonism in the rat vagus nerve preparation with a slope for the Schild plot that was not significantly different from 1 ($P>0.1$). The apparent $K_d$ values for capsazepine in assays on cultured DRG neurones and vagus nerve were not significantly different as shown by the extensive overlap in the 95% confidence limits. Thus these data indicate that capsazepine acts at a very similar, if not identical, binding site in both cultured neonatal rat cells and in acutely isolated tissue from adult animals.

The effects of RTX are very similar to those of capsaicin. Both agents act on the same population of DRG neurones with a similar mode of action (Winter *et al.*, 1990). In addition, the structural similarity between parts of the RTX and capsaicin molecules led to the suggestion that both

molecules act at the same cellular site (de Vries & Blumberg, 1989). However, RTX also shows considerable structural similarity to phorbol esters and has been reported to activate a protein kinase (Ryves *et al.*, 1989). It has therefore been possible to argue that RTX acts, at least in part, at a different site from capsaicin. The findings that capsazepine was a competitive antagonist of RTX and that essentially identical $K_d$ estimates for capsazepine were obtained with either RTX or capsaicin as the agonist (capsaicin 148 nM; RTX 107 nM, $^{86}Rb^+$ efflux assay, overlapping 95% confidence limits), argue that for $Rb^+$ efflux both agonists operate by binding to the same site. As capsaicin is known to activate ion channels in isolated patches of plasma membrane (Forbes & Bevan, 1988), the binding site for these agents must be in, or intimately associated with, the membrane.

The inhibition by capsazepine was specific to the capsaicin response. The responses to GABA, ATP and protons were not inhibited by high concentrations of capsazepine (10 μM). Similarly, capsazepine did not reduce the ion flux evoked by $K^+$ induced depolarization of either vagus nerve (guanidinium ions) or cultured DRG neurones ($^{86}Rb^+$). In these latter experiments only ion flux through slowly inactivating or non-inactivating voltage-sensitive channels would have been measured with the protocols used. Guanidinium ions are permeant through $Ca^{2+}$ channels (McCleskey & Almers, 1985) but not through delayed rectifier $K^+$ channels, whereas $Rb^+$ is highly permeant through $K^+$ channels (see Hille, 1984). The failure to inhibit [$^{14}$C]-guanidinium and $^{86}Rb^+$ fluxes suggests that capsazepine has little or no effect on these channel types. Of course, the use of these ions is not a definitive test for the activity of voltage-gated $Ca^{2+}$ or delayed rectifier $K^+$ channels, nevertheless, the lack of inhibition of the depolarization-induced ion flux shows that capsazepine does not act on a significant population of voltage-gated ion channels.

Ruthenium Red has been used previously as a capsaicin antagonist although it is unclear how it acts (see Amman & Maggi, 1991; Maggi, 1991 for reviews). It does not block the binding of RTX to DRG membranes (Szallasi & Blumberg, 1990) although it does inhibit the activity of capsaicin operated ion channels (Dray *et al.*, 1990b). Observations on single capsaicin-activated channels suggest that, unlike lanthanum, Ruthenium Red does not act as a simple open channel blocker (Dray *et al.*, 1990b). Our experiments indicate that Ruthenium Red is not a competitive antagonist of capsaicin. It depressed the maximum $^{45}Ca^{2+}$ accumulation evoked by capsaicin in cultures of DRG neurones, without any marked parallel shift in the (log-concentration)-response curves. This calcium accumulation involves not only calcium entry but also sequestration by intracellular compartments (see Wood *et al.*, 1988) and so the failure to observe any obvious competitive inhibition by Ruthenium Red could reflect actions at steps subsequent to ion entry. However, similar results were noted in efflux experiments made on vagus nerve preparations, which give a more direct measure of plasma membrane permeability. Together these experiments suggest that Ruthenium Red acts as a non-competitive capsaicin antagonist.

Other experiments have shown that the use of Ruthenium Red as a capsaicin antagonist has to be restricted to a narrow range of concentrations (0.1–10 μM, see Maggi, 1991). Even at these concentrations, it is not specific for capsaicin and can prevent primary afferent nerve activation induced by noxious heat (Amman *et al.*, 1990), toluene diisocyanate (Mapp *et al.*, 1991a), protons (Geppetti *et al.*, 1991) and prostacyclin ($PGI_2$) (Mapp *et al.*, 1991b). Similar concentrations of Ruthenium Red also affect some types of ion channels and receptors in a wide range of cell types (Weiss, 1977; Adams *et al.*, 1985; Tapia *et al.*, 1985; Ong *et al.*, 1987; Robertson & Wann, 1987; Davidson *et al.*, 1988; Grasso & Reichert, 1989; Williams *et al.*, 1990). Furthermore Ruthenium Red is known to inhibit intracellular $Ca^{2+}$ transport processes and thereby raise the level of intracellular free $Ca^{2+}$ in intact preparations (Rossi *et al.*, 1973; Raess & Vincenzi, 1980; Bernath & Vizi, 1987). Therefore, although it may be a useful tool, the lack of specificity of Ruthenium Red limits its use in the study of the action of capsaicin.

Our data indicate that capsazepine is a specific antagonist of the sensory neurone actions of capsaicin and RTX. In this respect it differs from Ruthenium Red, which has a non-competitive mode of action and can also interfere with the activity of other receptors and ion channels. At present it is unclear whether capsaicin and RTX simply mimic the actions of endogenous, structurally similar molecules or whether they activate an ion channel that is not normally operated in such a way. Capsazepine will be of use in addressing such a problem. In addition capsazepine provides us with a tool to dissect the specific, sensory neurone actions of capsaicin from the non-specific actions (see introduction) and so will allow us to investigate, more precisely, the physiological roles of capsaicin sensitive neurones. Already capsazepine has been shown to antagonize the actions of capsaicin *in vivo* (Dray *et al.*, 1991) and has been used to provide evidence that capsaicin exerts an anti-nociceptive effect by acting on specific receptors localized to sensory nerve fibres within the spinal cord (Dickenson & Dray, 1991).

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*(Received February 14, 1992*
*Revised June 1, 1992*
*Accepted June 16, 1992)*